Geographic variation in nrDNA and four cpDNA regions of *Juniperus excelsa* and *J. polycarpos* from Greece, Turkey, Lebanon and Azerbaijan

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ABSTRACT

DNA sequencing of nrDNA, plus four cp DNA regions: petN-psbM, trnS-tenG, trnD-trnT and trnL-trnF of *J. excelsa* and *J. polycarpos* from Azerbaijan, Greece, Lebanon and Turkey revealed that putative *J. excelsa* from Azerbaijan is *J. polycarpos* (= *J. excelsa* subsp. *polycarpos*). Two Lebanon *Juniperus* populations from Afqa (1306 m) and Wadi El Njass (2287 m), previously shown to be divergent in their microsatellites, were found to be *J. excelsa* and *J. polycarpos,* respectively. This is the first report of the occurrence of *J. polycarpos* in Lebanon. To aid comparisons, DNA sequences from *J. seravschanica* and *J. polycarpos* var. *turcomanica* and *J. procera* were included in the Bayesian analysis.
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KEY WORDS: *Juniperus excelsa, J. polycarpos* var. *polycarpos, J. polycarpos* var. *turcomanica, J. seravschanica,* DNA sequencing, nrDNA, petN-psbM, trnS-tenG, trnD-trnT, trnL-trnF.

Recently, Douaihy et al. (2011), using 3 microsatellites of putative *J. excelsa*, found the Nei genetic distance separated their 12 populations into 3 groups: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations (Fig. 1). PCO of the data removed 38.8% and 27.5% on the first two axes. Ordination clearly shows: Lebanon (Leb 1, 2, 4, 5), Lebanon (Leb 3, 6) and the Crimea, Cyprus, Greece and Turkey populations (Fig. 2). El Njass (Leb 3, 2287 m) and Aarsal (Leb 6, 2180 m) are from higher elevations in Lebanon. Examination of specimens (RPA) from Afqa, 1300 m and El Njass, 2287 m, found the leaves of Afqa plants had very fine, small leaves and were bluish green similar to *J. excelsa* from Greece. The leaves of the El Njass plants were larger, coarse and yellowish green similar to *J. polycarpos* from Armenia and *J. p.* var. *turcomanica* from Turkmenistan.

In a comprehensive study of the reproductive ecology of *Juniperus* in Lebanon, Douaihy et al. (2013) reported differences between the higher (El Njass, Aarsal) and lower (Afqa, etc.) populations in cones density classes, frequencies of adult and juvenile trees, and dioecious (El Njass, Aarsal) vs. monecious (Afqa, etc.) individuals. Interestingly, Adams (2014) describes *J. excelsa* as monoecious or dioecious and *J. polycarpos* as dioecious.

Juniperus excelsa M.-Bieb. grows from Greece to Turkey and perhaps as far east as Azerbaijan (Fig. 1) . Farjon (2005, 2010) treated *J. polycarpos, J. p.* var. *seravschanica* and *J. p.* var. *turcomanica* as *J. excelsa* subsp. *polycarpos*. However, Adams and Schwarzbach (2012) and Adams (2013), utilizing

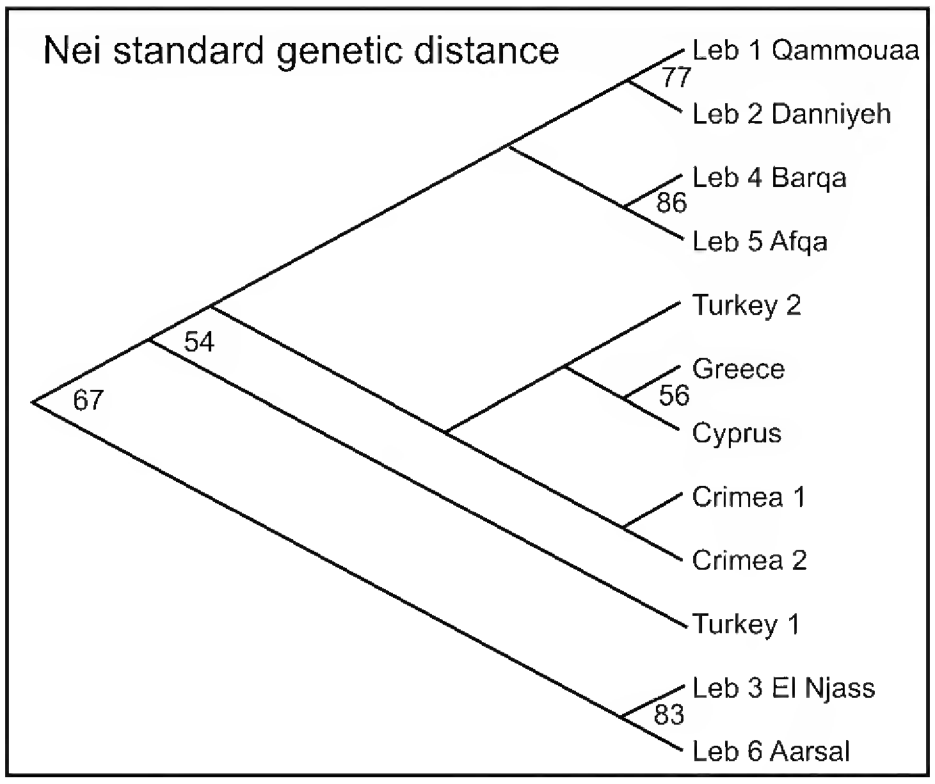

Figure 1. Nei distance diagram. Note the Lebanon (Leb 1, 2, 4, 5) at top and Leb 3, 6 at bottom.

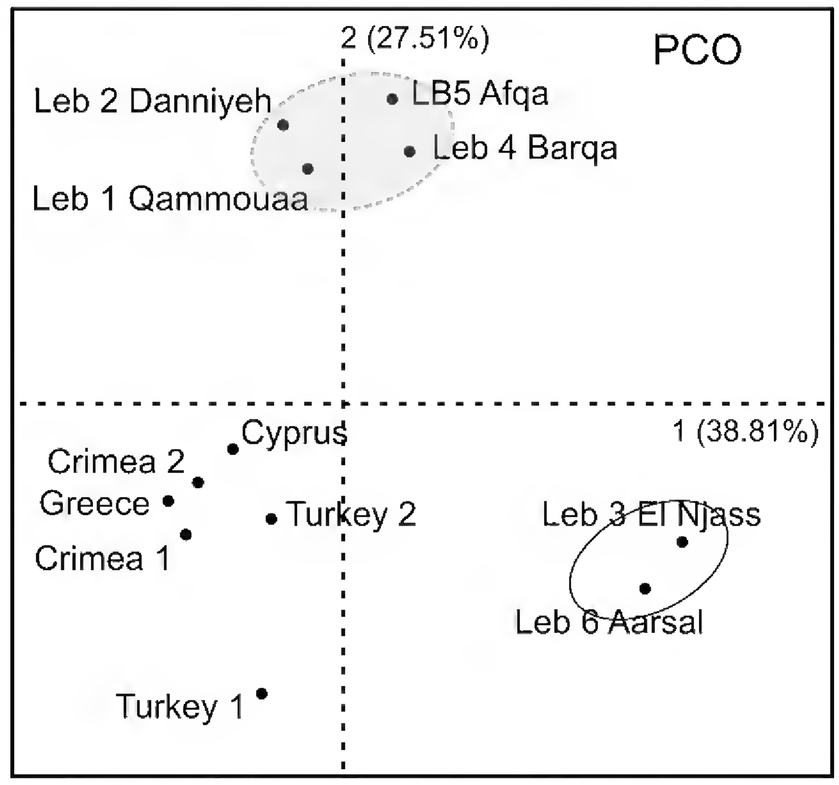

Figure 2. PCO, microsatellite data. *J. excelsa* is ordinated into 3 groups.

DNA sequence data, recognized *J. excelsa* in addition to *J. polycarpos, J. p.* var. *turcomanica* and *J. seravschanica.* Adams and Hojjati (2012) and Adams, Hojjati and Schwarzbach (2014), using sequences from 4 gene regions, failed to verify the occurrence of *J. excelsa* in Iran, but did find *J. polycarpos, J. p.* var. *turcomanica* and *J. seravschanica* in Iran. Putative *J. excelsa* from Qushchi, in extreme northwest Iran, had none or only one SNP difference compared with *J. polycarpos* var. *polycarpos* from Armenia and was concluded to be *J. polycarpos* (Adams and Hojjati, 2012).

The distributions of *J. excelsa* and *J. polycarpos* (stricto sensu) are shown in Figure 3. It is difficult to distinguish these taxa and they have been treated as conspecific (Farjon, 2005, 2010; Douaihy et al, 2011). The distribution of *J. excelsa* into Armenia, Azerbaijan and Iran has proved difficult to determine by modern methods of DNA sequencing and leaf essential oil data, due to the lack of access to these regions. Recently, materials were obtained of *J. excelsa/ J. polycarpos* from Lebanon and *J. excelsa/ J. polycarpos* from Azerbaijan. This afforded the opportunity to further examine geographic variation in the DNA sequences of both *J. excelsa* and *J. polycarpos*.

The purpose of the paper is to examine nrDNA, and 4 cp DNA regions: petN-psbM, trnS-trnG, trnD-trnT and trnL-trnF and report on variation in *J. excelsa* and *J. polycarpos*.

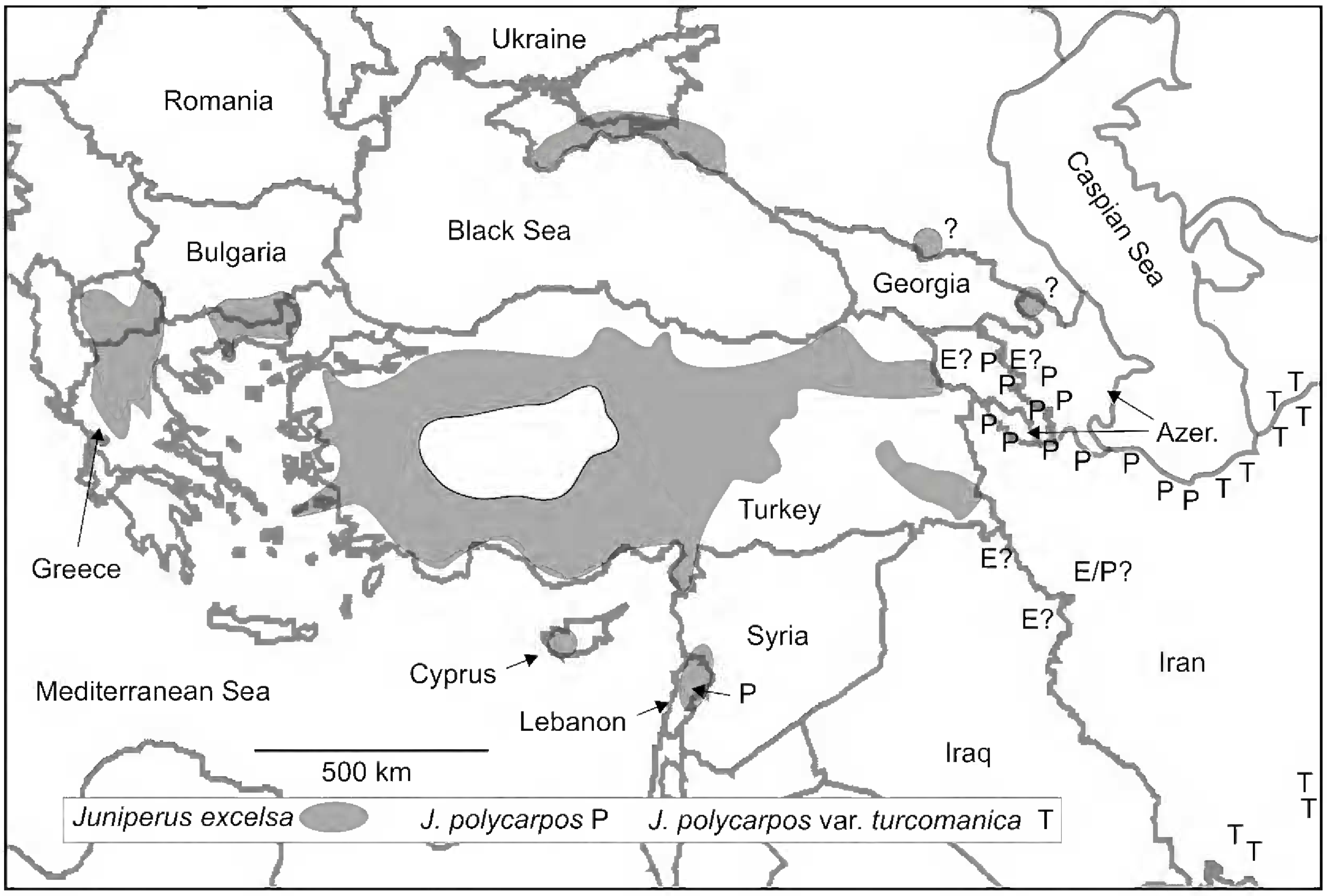

Figure 3. Distribution of *J. excelsa*, *J. polycarpos* var. *polycarpos* (P) and *J. p.* var. *turcomanica* (T) . Questionable locations of *J. excelsa* and *J. polycarpos* are indicated by E? and P? (modified from Adams, 2014).

MATERIALS AND METHODS

Plant material -

J. excelsa: Eskisehir, Turkey, 820m, *Adams 13193 (9433-9435),* Bulgaria, 356 m, *Adams 14056 (13720-13724), Alex Tashev, 2012-1-JE -5-JE*, 42° 01' 22.0" N; 24° 28' 03.1" E, Central Rhodopes, above the town of Kritchim, Reserve "Izgorialoto Gune"; Lemos, Greece, 1100m, *Adams 6031 (5983-5985, 5987*), Cyprus, *Adams 13487*, *A. K. Christou s.n.*, bulk 5 trees; Afqa, Lebanon, 1306 m, *Adams 14155-14157*, *Bouchra Douaihy 1-3*, 34° 04' 58.12"N, 35° 53' 08.52"E, 4 Nov 2013,

J. polycarpos: Armenia, Lake Sevan, 1900m, *Adams 13194 (8761-8763)*; Azerbaijan, 177-231m, *Adams 14162-14171, Vahid Farzaliyev 1-10*, 40° 44' 41.05" N; 47° 35' 19.14" E, Dec 2013; Lebanon, Wadi El Njass, 2287m, *Adams 14158-14161*, *Bouchra Douaihy 4-7*, 34° 20' 47.79"N, 36° 05' 45.54"E, 14 Nov 2013.

J. polycarpos var. *turcomanica*: *Adams 13197 (8758-90),* Kopet Mtns., Turkmenistan;

J. procera: *Adams 6184-6188*, Guder, Ethiopia;

J. seravschanica: *Adams 13195 (8483-85)*, Pakistan.

Voucher specimens deposited in the Herbarium, Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions.

Amplifications were performed in 30 μl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 μl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 μM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 μM each primer. See Adams,

Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010).

The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R6-1 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al., 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing nrDNA (ITS) and four cp regions petN-psbM, trnS-tenG, trnD-trnT and trnL-trnF yielded 4430 bp of data. The Bayesian consensus tree (Fig. 4) shows *J. seravschanica, J. polycarpos, J. p.* var. *turcomanica, J. procera* and *J. excelsa* in well-supported clades. All of the samples from Azerbaijan are closely nested with *J. polycarpos*, Armenia along with the El Njass, Lebanon (Adams 14161) sample (Fig. 4). Three other El Njass samples (*14158, 58, 60*) appear to be intermediate between *J. polycarpos and J. p.* var. *turcomanica* (Fig. 4). The three Afqa, Lebanon samples (*14155, 56, 57*) are in the clade with *J. excelsa* from Greece and Turkey (Fig. 2).

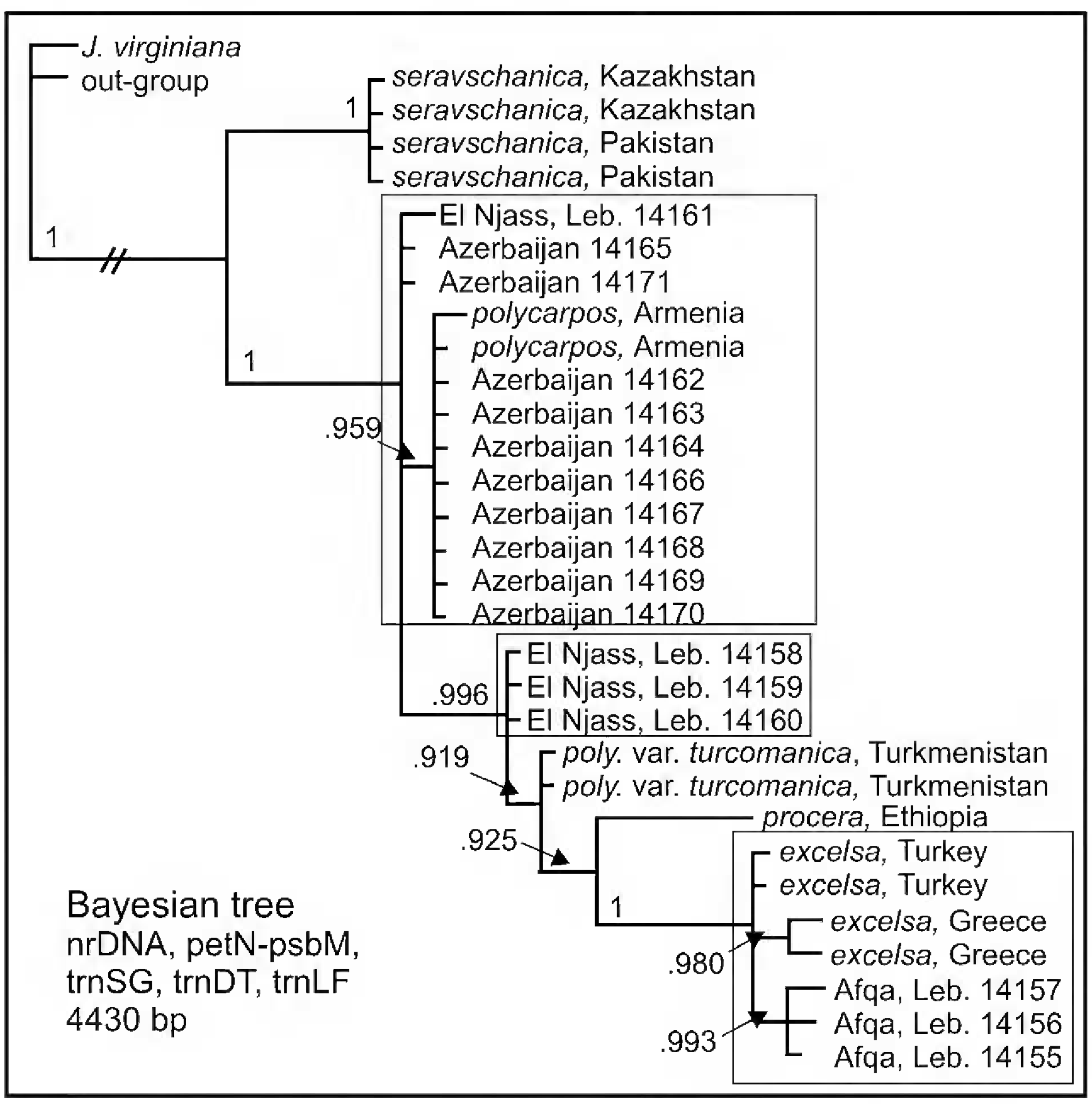

Figure 4. Bayesian tree based on nrDNA (ITS) and four cp regions: petN-psbM, trnS-tenG, trnD-trnT and trnL-trnF (4430 bp). Numbers at the branch points are posterior probabilities.

To examine the magnitude of the differences among the taxa, aligned sequences were examined for single nucleotide polymorphisms plus indels (= mutation events, MEs). Analysis revealed a total of 114 MEs, with 18 occurring only once and 96 occurring 2 or more times (i.e., multiple). Although the single MEs may be of interest in examining mutation rates, only the 96 multiple occurrence MEs were utilized in the construction of a minimum spanning network (Fig. 5). All the samples from Azerbaijan and El Njass, Lebanon are found between *J. polycarpos*, Armenia and *J. p.* var. *turcomanica*, Turkmenistan (Fig. 5). Three of the El Njass trees differ by only 1 ME from *J. p.* var. *turcomanica*. The fourth El Njass sample (14161) differs by only 1 ME from Azerbaijan samples (Fig. 5). Two Azerbaijan trees (14165, 14171) differ by 1 ME from the other 8 Azerbaijan samples (Fig. 5). Clearly the El Njass and Azerbaijan samples are *J. polycarpos*.

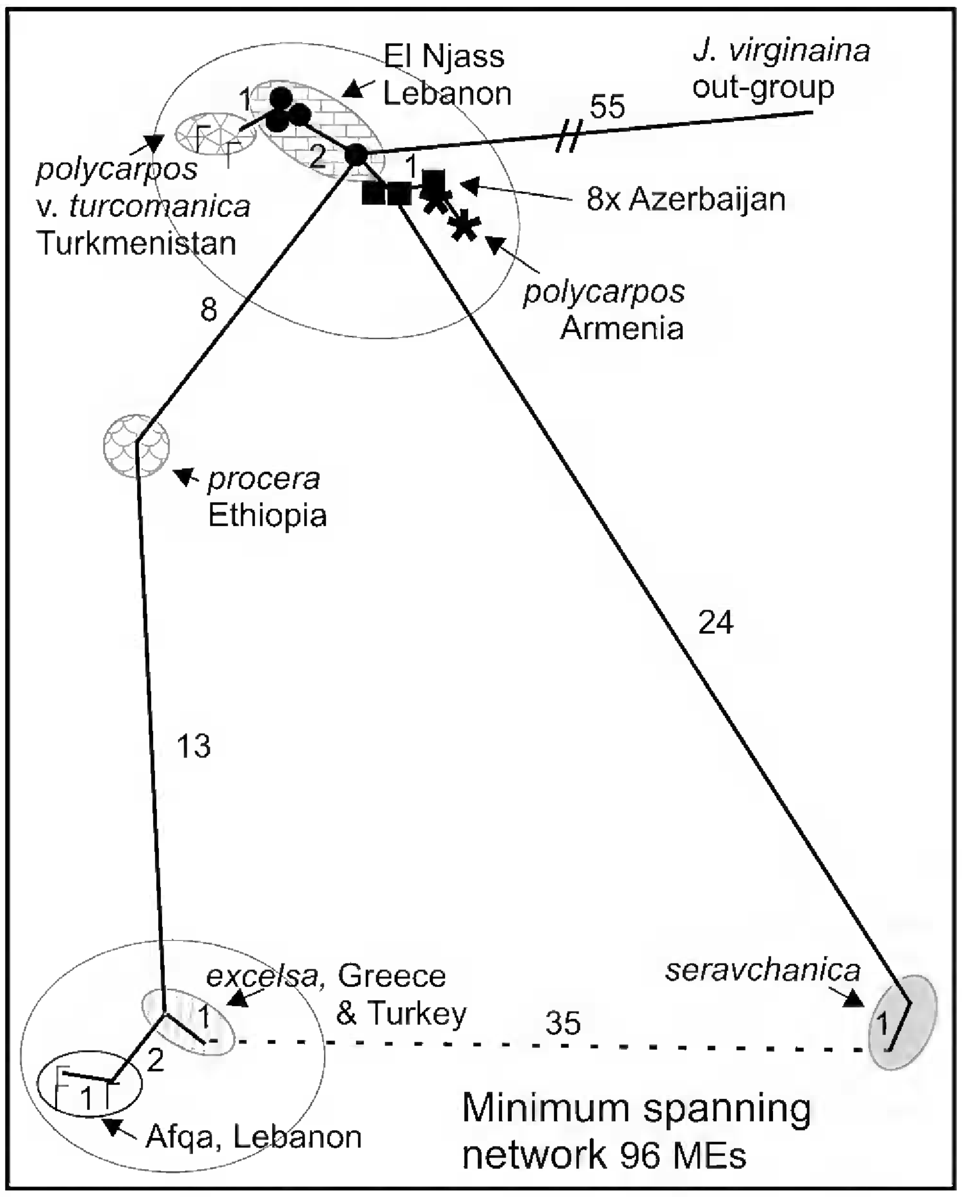

Figure 5. Minimum spanning network based on 96 MEs. Numbers next to links are the number of MEs separating the nodes. The dashed line is the shortest link between *J. excelsa* and *J. seravschanica* (35 MEs).

Juniperus procera differs by 8 MEs from *J. polycarpos* and 13 MEs from *J. excelsa* (Fig. 5), whereas *J. seravschanica* differs by 24 MEs from *J. polycarpos*. The three trees from Afqa, Lebanon differ by only 2 MEs from *J. excelsa*, Turkey (Fig. 5).

Overlaying a minimum spanning network onto a distribution map gives one a perspective of the geographic trends (Fig. 6). The Afqa, Lebanon *J. excelsa* population differs by 2 MEs from Eskisehir, Turkey, which in turn, differs by only 1 MEs from the Lemos, Greece population (Fig. 6). The other Lebanon populations that group with Afqa (Figs. 1, 2) are probably *J. excelsa*.

However, the Wadi El Njass, Lebanon (2287 m) population, although very near Afqa, is *J. polycarpos* and differs by 1 to 3 MEs from *J. p.* var. *turcomanica*, Turkmenistan and by 1 to 2 MEs from *J. polycarpos,* Armenia (Fig. 6). The *J. excelsa*, Afqa population is only about 100 - 150 km from other

J. excelsa populations (Fig. 6), but the Wadi El Njass, *J. polycarpos* population is 700 to 1000 km from the nearest *J. polycarpos* population. Yet, it differs by only 1 to 3 MEs.

The discovery of the *J. polycarpos*, Wadi El Njass population in Lebanon was unexpected. The trees at nearby Aarsal are probably *J. polycarpos* (Figs. 1, 2). Lebanon may have been a refugium for *J. polycarpos* during the Pleistocene ice age. This discovery also points to the need for more extensive field work on the isolated mountains of northern Syria, Iraq and Iran to determine if other disjunct populations of *J. polycarpos* (or *J. excelsa*) exist.

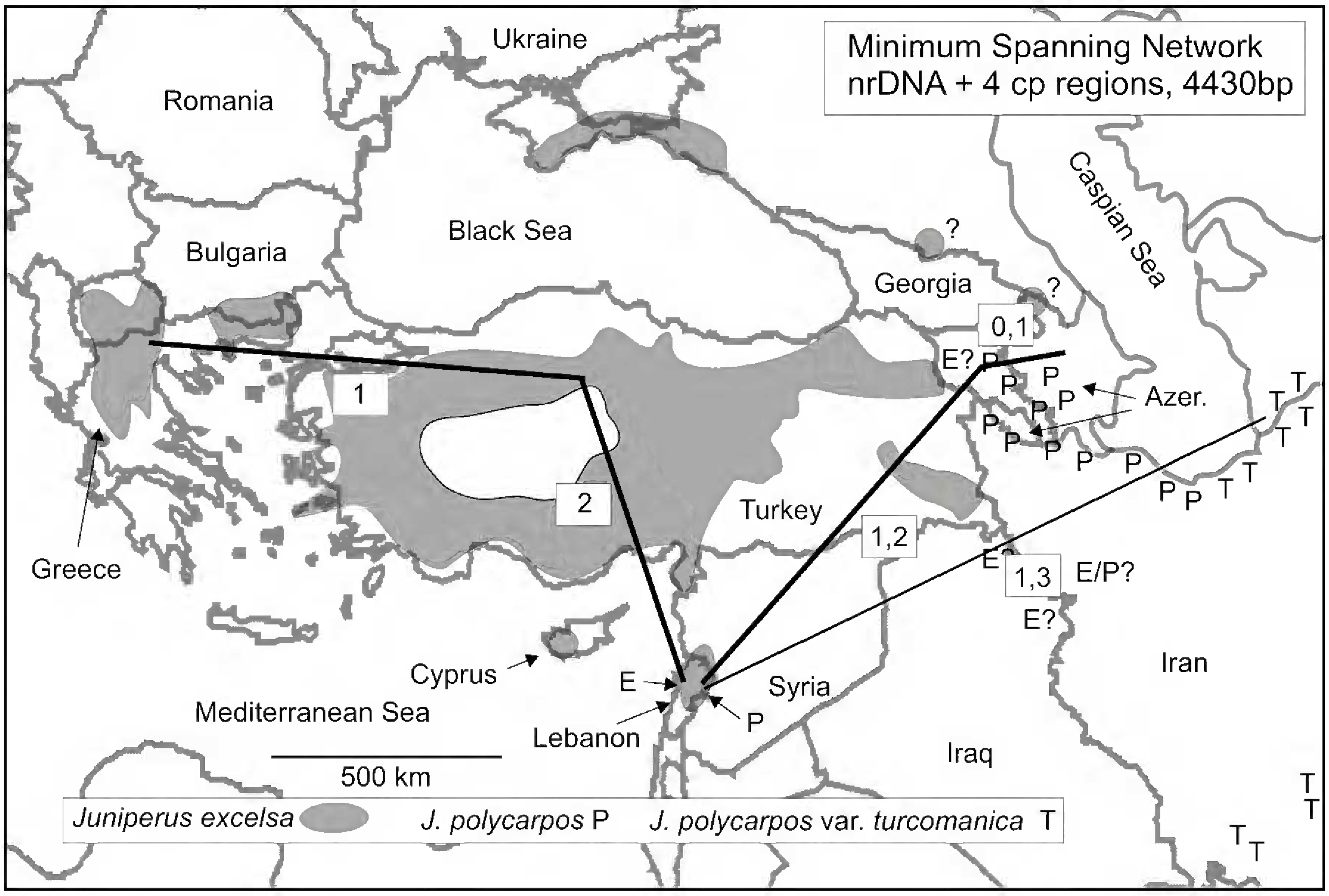

Figure 6. Minimum spanning network mapped onto the distributions of *J. excelsa and J. polycarpos*. Numbers next to lines are the number of MEs.

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